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## Function of the fully conserved residues Asp99, Tyr52 and Tyr73 in phospholipase A<sub>2</sub>

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In the active centre of pancreatic phospholipase A<sub>2</sub> His48 is at hydrogen-bonding distance to Asp99. This Asp–His couple is assumed to act together with a water molecule as a catalytic triad. Asp99 is also linked via an extended hydrogen bonding system to the side chains of Tyr52 and Tyr73. To probe the function of the fully conserved Asp99, Tyr52 and Tyr73 residues in phospholipase A<sub>2</sub>, the Asp99 residue was replaced by Asn, and each of the two tyrosines was separately replaced by either a Phe or a Gln. The catalytic and binding properties of the Phe52 and Phe73 mutants did not change significantly relative to the wild-type enzyme. This rules out the possibility that either one of the two Tyr residues in the wild-type enzyme can function as an acyl acceptor or proton donor in catalysis. The Gln73 mutant could not be obtained in any significant amounts probably due to incorrect folding. The Gln52 mutant was isolated in low yield. This mutant showed a large decrease in catalytic activity while its substrate binding was nearly unchanged. The results suggest a structural role rather than a catalytic function of Tyr52 and Tyr73. Substitution of asparagine for aspartate hardly affects the binding constants for both monomeric and micellar substrate analogues. Kinetic characterization revealed that the Asn99 mutant has retained no less than 65% of its enzymatic activity on the monomeric substrate *rac* 1,2-dihexanoyldithio-propyl-3-phosphocholine, probably due to the fact that during hydrolysis of monomeric substrate by phospholipase A<sub>2</sub> proton transfer is not the rate-limiting step. The Asp to Asn substitution decreases the catalytic rate on micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine 25-fold. To explain this remaining activity we suggest that in the mutant the Asn99 orients His48 in the same way as Asp99 orients His48 in native phospholipase A<sub>2</sub> and that the lowered activity is caused by a reduced stabilization of the transition state.

**Key words:** catalytic mechanism/phospholipase A<sub>2</sub>/protein folding/site-directed mutagenesis

### Introduction

The lipolytic enzyme phospholipase A<sub>2</sub> (PLA<sub>2</sub>) catalyses the hydrolysis of the 2-acyl ester linkage of 1,2-diacyl-*sn*-3-phosphoglycerides in a calcium-dependent reaction (van Deenen and de Haas, 1964). In the pancreas the enzyme occurs as a zymogen which is activated by trypsin upon secretion into the duodenal tract, where it serves a digestive function (de Haas *et al.*, 1968; Dutilh *et al.*, 1975). The catalytic properties of precursor and activated PLA<sub>2</sub>, with respect to monomeric substrates, are quite similar. However, when the substrate is present in an aggregated form, such as micelles, there is a large increase in enzymatic activity of the mature enzyme, but not of its zymogen (Pieterse *et al.*, 1974). Chemical modification and X-ray crystallography

studies (Verheij *et al.*, 1980; Fleer *et al.*, 1981; Dijkstra *et al.*, 1983) have indicated the importance of His48, Asp99 and Asp49, the latter residue as a Ca<sup>2+</sup>-binding ligand, for the enzymatic activity of pancreatic PLA<sub>2</sub>. Based on these data a general catalytic mechanism has been proposed (Verheij *et al.*, 1980), in which the aspartate–histidine couple and a water molecule serve as a catalytic triad (Figure 1). The presence of the Asp–His couple in the active site of PLA<sub>2</sub> suggests a similarity to the proton relay system of the serine esterases (Stamato *et al.*, 1986). This comparison is not unrealistic, because both phospholipase and the proteolytic serine esterases are able to hydrolyse carboxylic acid esters. In the widely studied serine proteases, the active site histidine of the pair is coupled to the nucleophilic serine residue and stabilizes the oxyanionic form of the serine by acting as a base. In the so-called charge relay system the catalytic Asp residue accepts a proton from the His, in this way stabilizing the monoprotonated His. Recently it was suggested that the active site aspartate contributes to the catalytic efficiency by stabilizing the positively charged form of the histidine residue in the transition state (Warshel *et al.*, 1989) and by anchoring it in the most favourable orientation for acid/base catalysis (Sprang *et al.*, 1987). In the three-dimensional structure of PLA<sub>2</sub> no serine is present near His48, but a water molecule, 3.1 Å from the N1 nitrogen of His48, which lies in the plane of the imidazole ring, could perform the nucleophilic function. Thus, the ester hydrolysis by PLA<sub>2</sub> seems to be comparable with the deacylation step in the serine proteases, the difference being that in the latter case the substrate is covalently bound to the enzyme.

According to the three-dimensional structure, the Asp99 residue forms four hydrogen bonds in the interior of the enzyme. The O<sub>δ1</sub> atom can accept hydrogen bonds from Tyr73 and from a water molecule, which in turn is H-bonded to the NH<sub>3</sub><sup>+</sup> group of Ala1 and to Tyr52. The O<sub>δ2</sub> atom of Asp99 can accept hydrogen bonds of the H atom of the N3 of His48 and of Tyr52 (Figure 1). The two tyrosines at positions 52 and 73 are fully

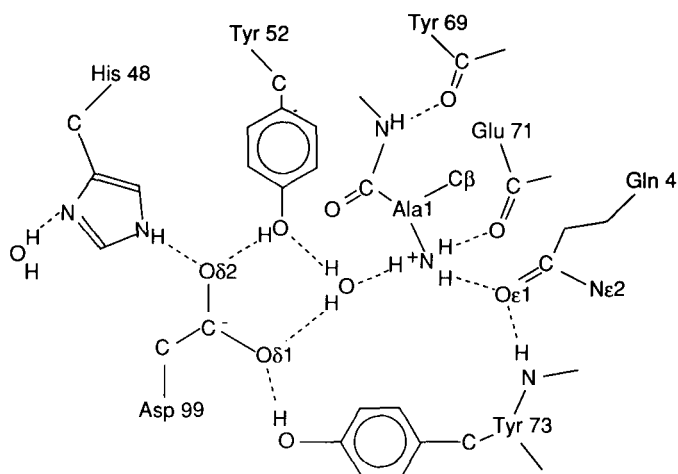


Fig. 1. Hydrogen-bonding network around the active site and the N-terminus of porcine PLA<sub>2</sub> (from Renetseder, 1986).

conserved in all 79 known sequences of pancreatic, snake venom and intracellular mammalian PLA<sub>2</sub> (for a comparison of 50 sequences see van den Bergh *et al.*, 1989). The conservation of these two tyrosines and their location close to His48 and Asp99 has prompted Brunie *et al.* (1985) to propose that these residues belong to the active site and could serve a catalytic function as an acyl acceptor or as a general acid. However, experimental evidence for such a function has not been obtained yet. Our purpose is to probe the functions of Asp99 and the two conserved Tyr residues by site-directed mutagenesis. All mutations are applied in a PLA<sub>2</sub> mutant, in which a surface loop was deleted; this mutant is denoted as  $\Delta 62-66$  PLA<sub>2</sub>. In contrast to native porcine pancreatic PLA<sub>2</sub> this  $\Delta 62-66$  PLA<sub>2</sub> mutant readily crystallizes and shows increased catalytic activities compared with native enzyme (Kuipers *et al.*, 1989a). In this way the kinetic and structural characterization of the mutant enzymes, which are anticipated to have considerably lower catalytic activities than the  $\Delta 62-66$  enzyme, can be facilitated. The mutant enzymes will be denoted by a ' $\Delta$ ', followed by the amino acid residue substitution.

To provide an empirical test for the contribution of the Asp99 residue to PLA<sub>2</sub> catalysis, this residue was mutated to the isosteric asparagine. The Tyr52 and Tyr73 residues were each replaced separately by either a Phe or a Gln. The Phe mutation will retain the aromatic character of the residue, but remove possible hydrogen bonds to Asp99 and the water molecule. The mutants with Gln at positions 52 or 73 are still able to form hydrogen bonds, but have lost their aromatic characteristics. To characterize these PLA<sub>2</sub>s, their affinities for monomeric and micellar substrate analogues were determined, and their catalytic properties towards monomeric and micellar substrates were investigated. The results of the characterization of these mutant enzymes will be discussed in relation to a possible role in catalysis, folding and/or stability.

## Materials and methods

### Construction of mutant phospholipases

*Escherichia coli* K-12 strain PC 2494 [ $\Delta(lac-pro)$ , *supE*, *thi/F'* *traD36*, *proA*<sup>+</sup>*B*<sup>+</sup>, *lacI*<sup>q</sup>, *LacZ*  $\Delta$ M15, Phabagen collection, Utrecht] was used for plasmid constructions and as a host for M13-derived vectors. HB 2154 [*ara*,  $\Delta(lac-pro)$ , *thi/F'*, *proA*<sup>+</sup>*B*<sup>+</sup>, *laqI*<sup>q</sup>, *lacZ*  $\Delta$ M15, *mutL::Tn10*] (Carter *et al.*, 1985) was used as recipient strain in mutagenesis experiments. Substitutions were introduced in porcine pancreatic PLA<sub>2</sub> lacking a surface loop ( $\Delta 62-66$ ) (Kuipers *et al.*, 1989a). After subcloning the *pro*- $\Delta 62-66$ -DNA into the M13 vector mp8, mutagenesis was performed according to the gapped-duplex method (Kramer *et al.*, 1984). The mutagenic oligonucleotides 5' CATTTCGGTT\*ACAGTTACAT\*ATGAAGGC 3' (Asn99), 5' GGCATCTCTA\*A\*AGCAGTTGTCG 3' (Phe52), 5' GGC-ATCTCTT\*TG\*GCAGTTGTCG 3' (Gln52), 5' GAGTAG-GAGA\*AGCTTTCGGT 3' (Phe73) and 5' TGAGTAGGAT\*TG\*GCTTTCGGT 3' (Gln73), were used for site-directed mutagenesis. In these oligonucleotides, synthesized on a Biosearch 6800 DNA synthesizer, an asterisk denotes a change of the preceding nucleotide compared with the cDNA sequence of the wild-type PLA<sub>2</sub>. Screening of mutants was performed by dideoxy-sequencing after plaque purification. From the sequenced DNA a *KpnI*–*HindIII* fragment (or a *KpnI*–*BglII* fragment in the case of Phe73) containing the mutated  $\Delta 62-66$ -DNA, was cloned into the expression vector pOK13 (de Geus *et al.*, 1987; Kuipers *et al.*, 1989b). After transformation and expression in

*E. coli* K-12 strain MC4100 (Casadaban, 1976), containing plasmid pCI857, mutant phospholipases were obtained by tryptic cleavage of reoxidized fusion protein (de Geus *et al.*, 1987) and purified by CM–cellulose chromatography at pH 5 and 6. Final purification to homogeneity was achieved on DEAE–cellulose at pH 8. After purification residue substitutions were confirmed by amino acid analyses. For amino acid analyses, samples were hydrolysed *in vacuo* for 24 h at 110°C in 6 N HCl. Analyses were carried out on an LKB 4151 alpha plus analyser. The values for Thr and Ser were corrected for 5 and 10% loss respectively. Protein concentrations were calculated from the absorbance at 280 nm, for which were used  $E_{1\text{ cm}}^{1\%}$  values of 13.0 for  $\Delta$ Y52F,  $\Delta$ Y52Q and  $\Delta$ Y73F and 14.0 for  $\Delta 62-66$  and  $\Delta$ D99N PLA<sub>2</sub>.

### Phospholipids

The 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (diC8PC) used in this study was obtained after reacylation of *sn*-glycerol-3-phosphocholine (Cubero Robles and van den Berg, 1969). Racemic 1,2-dihexanoyldithio-propyl-3-phosphocholine (diC6dithioPC) was synthesized from the corresponding mercaptan by acylation of the SH-groups with acyl chloride and phosphorylation of the hydroxyl group (Volwerk *et al.*, 1979). *n*-Dodecylphosphocholine (C12PN) and *n*-hexadecylphosphocholine (C16PN) were prepared as described by van Dam-Mieras *et al.* (1975).

### Phospholipase assays

Quantitative measurements with micelles of diC8PC as the substrate were carried out with a titrimetric assay at pH 8 in the presence of 1 mM borate, 25 mM CaCl<sub>2</sub> and 100 mM NaCl at 25°C. Activities on monomeric diC6dithioPC were determined at pH 8, with 5,5'-dithiobis-[2-nitrobenzoic acid] (DTNB) as a chromogenic reagent in the presence of 100 mM NaCl, 100 mM CaCl<sub>2</sub> and 100 mM Tris, as described previously (Volwerk *et al.*, 1979).

### Direct binding of PLA<sub>2</sub> to monomers and micelles

The affinities of PLA<sub>2</sub> for monomers and micelles were determined by following the increase of tryptophan fluorescence upon addition of increasing concentrations of the non-hydrolysable substrate analogues *n*-dodecylphosphocholine (CMC 1.3 mM) for monomer binding and *n*-hexadecylphosphocholine (CMC 10  $\mu$ M) for binding to micelles. Spectra were recorded at 25°C with a Perkin-Elmer LS-5 Luminescence Spectrometer. The excitation was performed at 280 nm and the emission spectra were recorded from 310 to 370 nm; slit widths were 5 nm. Assays were performed in a buffer containing 100 mM NaAc, 50 mM CaCl<sub>2</sub> and 100 mM NaCl at pH 6.0. Protein concentrations used were 7–8  $\mu$ M. From saturation curves obtained with lipid monomers, a  $K_d$  value can be derived directly. The data concerning micelle binding were analysed in terms of the binding of the enzyme to a theoretical lipid particle consisting of *N* monomers with a dissociation constant  $K_d$ . As has been discussed extensively by de Araujo *et al.* (1979), the  $N \cdot K_d$  value is the experimental concentration at which 50% of the enzyme is saturated with micelles.

## Results

### Production of PLA<sub>2</sub> with mutations at positions 52, 73 or 99

For the expression of (mutant) phospholipases use was made of an expression system in which the protein is produced in the form of a fusion protein with *cro-LacZ* (de Geus *et al.*, 1987). Subsequently the fusion protein is folded and the disulphide

bridges of PLA<sub>2</sub> are formed *in vitro*. In the next step PLA<sub>2</sub> is liberated from the resulting reoxidized hybrid protein by limited trypsinolysis. For native PLA<sub>2</sub> this procedure yields the amount of active PLA<sub>2</sub> corresponding to what can be predicted from the amount of fusion protein and therefore it was concluded (de Geus *et al.*, 1987) that both the folding and the cleavage reaction are nearly quantitative. In the present study the production and purification of the  $\Delta 62-66$  and the mutants were established by the same procedure yielding similar amounts (50–70 mg per 10 l of culture) of pure  $\Delta 62-66$ ,  $\Delta Y52F$  and  $\Delta Y73F$ , as had been obtained before with native PLA<sub>2</sub>. The yield of the  $\Delta D99N$  and the  $\Delta Y52Q$  mutants after cleavage of reoxidized fusion protein by trypsin was only 20% of the usual amount, whereas the  $\Delta Y73Q$  mutant could not be obtained at all, despite the fact that the yield of fusion protein was similar for all mutants. A possible explanation for the low yield of the  $\Delta D99N$ ,  $\Delta Y52Q$  and  $\Delta Y73Q$  mutant enzymes could be that the *in vitro* refolding of the  $\Delta D99N$  and  $\Delta Y52Q$  mutants was seriously affected. The presence of mismatched disulphide bonds would make the scrambled phospholipase-like structures susceptible to degradation by trypsin, whereas correctly folded (native) PLA<sub>2</sub> is quite stable in the presence of trypsin. Indeed SDS–PAGE analyses of trypsin-incubated reoxidized fusion proteins of wild-type,  $\Delta 62-66$ ,  $\Delta D99N$ ,  $\Delta Y52Q$  and  $\Delta Y73Q$  PLA<sub>2</sub> indicated the susceptibility of the latter three proteins. After incubation with trypsin the amounts of released  $\Delta D99N$  and  $\Delta Y52Q$  PLA<sub>2</sub> were lower than normal, whereas  $\Delta Y73Q$  PLA<sub>2</sub> was virtually undetectable (data not shown).

#### Interaction of $\Delta 62-66$ , $\Delta D99N$ , $\Delta Y52F$ , $\Delta Y73F$ and $\Delta Y52Q$ mutant PLA<sub>2</sub>s with monomeric and micellar substrate analogues

After production and purification of the mutant enzymes, it was of interest to investigate the influence of the mutations on the affinities for monomeric and micellar substrate analogues. The three-dimensional structure of PLA<sub>2</sub> indicates that the residues at positions 73 and 99 are buried in the interior of the protein. Residue 52 is somewhat more exposed and might be able to have an interaction with a monomeric substrate (analogue) molecule. Because of their buried character none of these residues is expected to be involved directly in binding to lipid–water interfaces. The results of the direct binding experiments of the four mutant enzymes and  $\Delta 62-66$  with C12PN monomers and with C16PN micelles are given in Table I. The  $\Delta D99N$  mutant displayed an affinity twice as high for C12PN monomers as the parent  $\Delta 62-66$ . Since the main effect of this mutation is the removal of one negative charge in the active site, a diminished electrostatic repulsion between this residue and the phosphate of the C12PN monomer could be responsible for the slightly increased affinity of the  $\Delta D99N$  mutant for monomers. The affinity for monomers was not changed in the case of the  $\Delta Y52F$  and  $\Delta Y73F$  mutants, whereas the  $\Delta Y52Q$  mutant exhibited a slightly lower affinity for monomers. These results indicate that the residues at positions 52, 73 and 99 are probably not directly involved in substrate binding via hydrogen bonding, although, given the lower affinity of  $\Delta Y52Q$  PLA<sub>2</sub> for monomers, a contribution of Tyr52 or Phe52 via hydrophobic interaction with the monomeric substrate molecule cannot be excluded. The replacement of Tyr52 or Tyr73 by a Phe hardly affected the enzymes affinity for C16PN micelles. Only the affinities of the  $\Delta Y52Q$  and the  $\Delta D99N$  mutants were moderately diminished, relative to the  $\Delta 62-66$  mutant. It can be concluded that the binding capacities both for monomeric and for micellar substrate analogues are preserved in all mutants.

**Table I.** Affinities of  $\Delta 62-66$  and four mutant PLA<sub>2</sub>s for a monomeric and a micellar substrate analogue

Enzyme	C12PN (monomers) $K_d$ (mM)	C16PN (micelles) $N \cdot K_d$ ( $\mu$ M)
$\Delta 62-66$	0.8	140
$\Delta D99N$	0.4	320
$\Delta Y52F$	0.8	172
$\Delta Y73F$	0.8	118
$\Delta Y52Q$	2.1	396

C12N stands for *n*-dodecylphosphocholine and C16PN represents *n*-hexadecyl-phosphocholine. Accuracy was ~10% for each given value, except for the  $K_d$  of  $\Delta Y52Q$  on C12PN, which exceeds the CMC of C12PN considerably, yielding an accuracy of ~40%.

**Table II.** Enzymatic activities of  $\Delta 62-66$  and four mutant PLA<sub>2</sub>s on a monomeric and on a micellar substrate

Enzyme	diC6dithioPC (monomers) $k_{cat}/K_m$ (s/M) $10^{-3}$	diC8PC (micelles)		
		$V_{max}$ ( $\mu$ mol/min/mg)	$K_m$ (mM)	$k_{cat}/K_m$ (s/M) $10^{-3}$
$\Delta 62-66$	1.64	4310	2.8	366
$\Delta D99N$	1.07	172	3.7	11.1
$\Delta Y52F$	1.61	4750	2.5	452
$\Delta Y73F$	1.31	4360	2.0	519
$\Delta Y52Q$	0.26	39	5.4	1.7

Accuracy was ~10% for each given value.

#### Enzymatic activities of $\Delta 62-66$ , $\Delta D99N$ , $\Delta Y52F$ , $\Delta Y73F$ and $\Delta Y52Q$ mutant PLA<sub>2</sub>s on monomeric and micellar substrates

The enzymatic activities of the mutant enzymes were determined with the monomeric substrate diC6dithioPC (Table II). The specificity constants for  $\Delta 62-66$ ,  $\Delta Y52F$  and  $\Delta Y73F$  PLA<sub>2</sub>s fall in the same range. For the  $\Delta Y52Q$  mutant the specificity constant is significantly lower, whereas the substitution of asparagine for aspartate has only limited effects on the specificity constant for the monomeric substrate.

When looking at the data for the hydrolysis of micelles of dioctanoylleicithin (Table II) it is clear that the mutations Y52F and Y73F do not exert any significant effect on the activities, nor on the  $K_m$  values. Again a significant drop in activity and in substrate affinity was observed for the mutation Y52Q. These observations underline the importance of the presence of an aromatic residue at position 52 and show that a phenolic hydroxyl at positions 52 and 73 is not a requirement for enzymatic activity. Although the  $K_m$  values of  $\Delta D99N$  and of  $\Delta 62-66$  PLA<sub>2</sub> differ only slightly, the  $\Delta D99N$  has a 25-fold decreased activity compared with  $\Delta 62-66$  PLA<sub>2</sub>. These results suggest that in the  $\Delta D99N$  mutant the loss in activity is due to a reduced stabilization of the transition state, but that the active site histidine remains oriented, leaving its imidazole ring in the correct tautomeric state for acid/base catalysis.

#### Discussion

Previous papers from this laboratory have dealt with PLA<sub>2</sub> mutants which had in common that point mutations, or even a loop deletion of five amino acids in combination with three additional mutations, were applied at the surface of the enzyme (Kuipers *et al.*, 1989a,b, 1990). With these mutants no serious problems in their production, refolding and purification were ever met. In the present paper enzymes are described that have

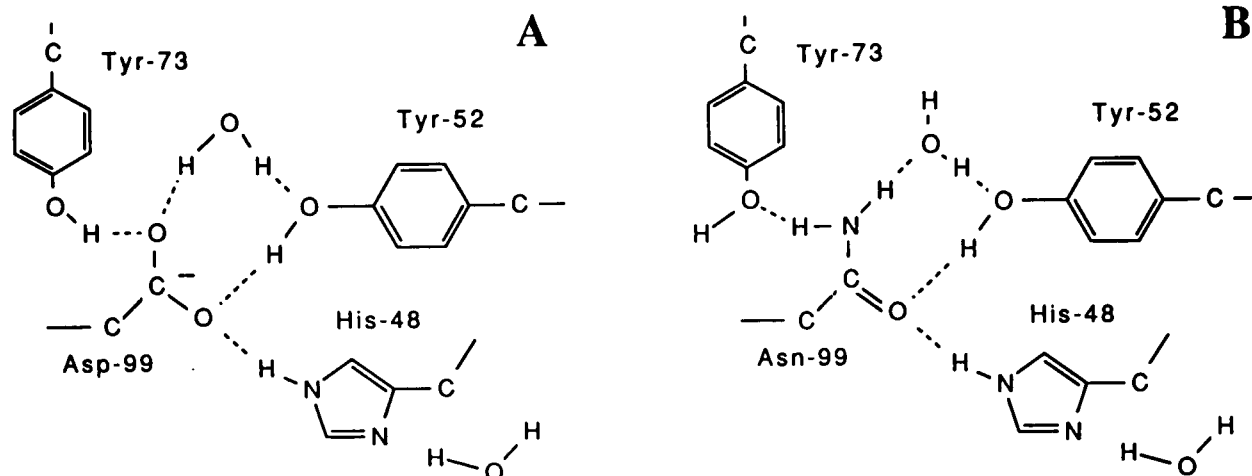


Fig. 2. (A) Hydrogen-bonding network in the active centre of native porcine phospholipase  $A_2$  (Dijkstra *et al.*, 1983). (B) Proposed hydrogen-bonding network in  $\Delta D99N$   $PLA_2$ .

mutations in the interior of the protein, and, perhaps due to this location, difficulties were encountered with the production of some of these mutants. Instability of proteins with mutations of internal residues is not unprecedented in protein engineering literature. For instance, it was reported recently that R35K and R87K mutations in the active site of staphylococcal nuclease disrupt the conformation of the active site, thereby reducing the catalytic efficiencies by a factor of  $10^4$  to  $10^5$  respectively (Pourmotabbed *et al.*, 1990). Furthermore, it has been shown that the interior packing and hydrophobicity of amino acid residues has a great influence on the stability of a protein (Sandberg and Terwilliger, 1989). In another study it was shown that weakly polar interactions between aromatic side chains of oligopeptides can stabilize their folding. The aromatic groups of globular proteins display a similar behaviour and thereby contribute to the stability of the three-dimensional structure of proteins (Burley and Petsko, 1989). In our case, the expression of all mutant proteins as fusion proteins in *E. coli* K-12 did not give rise to any difficulties, as the yield of inclusion bodies was equal for all mutants. However, after reoxidation and tryptic activation, the yields of  $\Delta D99N$  and  $\Delta Y52Q$  were considerably lower than those of  $\Delta Y52F$  and  $\Delta Y73F$ , while mutant  $\Delta Y73Q$  could not be obtained at all. It was shown by SDS-PAGE that, following limited trypsinolysis, a significant fraction of the reoxidized  $\Delta Y52Q$  and  $\Delta Y73Q$  mutant fusion proteins were degraded to peptides smaller than  $PLA_2$ , and that the remaining  $PLA_2$  band is about as stable as native  $PLA_2$ . The most straightforward explanation of this increased susceptibility to trypsin is that these mutants were only in part correctly folded. The incorrectly folded fraction is destroyed by the action of trypsin and the correctly folded fraction remains intact during prolonged periods of trypsinolysis. The substitution of Tyr by Gln or Phe results in the potential loss of hydrogen bonds. This could have significant structural effects as well as reducing the folding efficiency or stability. The fact that introduction of Gln had pronounced effects, whereas no problems occurred with the Tyr to Phe mutations at positions 52 and 73, which retain the aromatic character of the residue, suggests that the stacking of aromatic residues which has been reported for many proteins (Burley and Petsko, 1989), plays a role in  $PLA_2$  as well. Such a suggestion fits the observation that in the  $^1H$ -NMR spectrum of  $PLA_2$  the signal of the C2 proton of His48 shows a large

chemical shift (Aguilar *et al.*, 1979) probably due to the ring current of the nearby Tyr52. Also in the three-dimensional structure of  $PLA_2$  a close stacking of these two residues at an angle of  $80^\circ$  is observed suggesting a possible function of the aromatic ring of Tyr52 (or Phe52) in stabilizing the orientation of His48.

The fact that the mutants  $\Delta Y52F$  and  $\Delta Y73F$   $PLA_2$ s are nearly indistinguishable from the parent  $\Delta 62-66$   $PLA_2$  indicates that their hydroxyl groups are neither involved in substrate binding via hydrogen bonding nor in the true catalytic steps as acyl acceptor or as general acid or base. The small loss in affinity which is observed when the Tyr at position 52 is replaced by a Gln might indicate a limited hydrophobic interaction between Tyr52 and one of the acyl chains of the substrate. Because of their buried character it is hard, however, to envisage a direct participation of either Tyr52 or Tyr73 in interface recognition and binding. The observed moderate loss in affinity of mutant  $\Delta Y52Q$  for interfaces could simply be the result of a less optimal packing around the active site resulting in a slightly disturbed lipid binding domain.

The question remains what other possible function could then be ascribed to the fully conserved tyrosines. The kinetic results can be understood better if one assumes that Tyr52 and Tyr73 serve a structural rather than a catalytic function. The presence of aromatic groups at positions 52 and 73 in the interior of the protein might be necessary to form and maintain the hydrophobic wall of the active site. The close stacking of the aromatic ring of residue 52 with His48 could also contribute to a stabilization of the active site conformation. The hydrogen bonds of the two tyrosines to Asp99 and the water molecule could help in the stabilization of the enzyme, but removal of one of them by mutation of Tyr to Phe is not disastrous for the stability as long as an aromatic—or perhaps a large hydrophobic—residue is present. However, when a gap is created in the interior of the enzyme by substituting the much smaller Gln residue for Tyr, the enzyme becomes more unstable or could be hampered in its folding process which might lead to an incorrect conformation.

To elucidate the role of the highly conserved aspartate residue in the active centre of porcine pancreatic  $PLA_2$ , we replaced Asp99 by Asn. As could be expected for such an internal residue, the substitution in the  $\Delta 62-66$   $PLA_2$  has only limited effects on the affinity for monomeric and for micellar substrates and

substrate analogues. The effect of the mutation on the enzymatic hydrolysis of monomeric substrates is surprisingly small, since the  $\Delta D99N$  enzyme still possesses 65% of the  $\Delta 62-66$  activity (Table II) which seems to be in conflict with the proposed role in catalysis of Asp99 (cf. Figure 1). Besides the enzymatic activities on monomeric substrates, the activities of the  $\Delta 62-66$  and the  $\Delta D99N$  PLA<sub>2</sub> on zwitterionic aggregated phospholipids were determined. The substitution of asparagine for aspartate decreased the hydrolysis rate of dioctanoyllecithin by the mutant enzyme to ~4% compared with the  $\Delta 62-66$  PLA<sub>2</sub>. The latter reduction in catalytic efficiency is of the magnitude which has been predicted for such a substitution in the catalytic Asp-His-Ser triad of serine esterases (Fersht, 1985; Warshel *et al.*, 1989). In evaluating the discrepancy in these percentages of monomeric and micellar activities one should keep in mind that the 4% value still represents a turnover number of 41/s, whereas the 65% monomer hydrolysis corresponds to a turnover number of 1.3/s only. The latter value is so low that proton transfer from the attacking water molecule to His48 and from His48 to the leaving glycerol might not be rate limiting. For the hydrolysis of monomeric substrates it has indeed been observed that the reaction occurs as quickly in D<sub>2</sub>O as in H<sub>2</sub>O (J.J. Volwerk and H.M. Verheij, unpublished results). For the hydrolysis of micellar dioctanoyllecithin by  $\Delta 62-66$  the turnover number is as high as 1600/s and proton transfer to and from His48 may be an important factor in catalysis, thus explaining the sensitivity of the rate constant for the substitution of Asn for Asp.

Although our data for the substitution of asparagine for aspartate fit the theoretical predictions, the observed 25-fold reduction is low compared with values of ~25 000 for analogous substitutions in subtilisin and in rat pancreatic trypsin (Carter and Wells, 1988; Craik *et al.*, 1987). For the D102N trypsin mutant X-ray crystallographic studies (Sprang *et al.*, 1987) showed that the imidazole ring of His57 is free to rotate to the more favoured *trans* conformation, away from the catalytic site. So it may be assumed that the decreased serine nucleophilicity in D102N trypsin results from the lack of a base in the active site to accept the serine hydroxyl proton. His57 does not act as a base in this mutant, because it exists in the incorrect tautomer. In order to try to understand better the different effects of the Asp to Asn mutation in trypsin and in PLA<sub>2</sub>, a careful comparison of the positions of the amino acid side chains in the active centres of both trypsin and PLA<sub>2</sub> was made. In PLA<sub>2</sub>, one of the carboxylate oxygen atoms of Asp99 accepts hydrogen bonds from both the hydroxyl group of Tyr73 and a water molecule (Figure 2A), whereas in trypsin this hydrogen bonding results from the main-chain amide groups of residues 56 and 57. The other carboxylate oxygen atom of Asp99 in PLA<sub>2</sub> accepts hydrogen bonds from the N3 atom of His48 and the hydroxyl group of Tyr52, comparable with the hydrogen bonding by the N1 atom of His57 and the hydroxyl group of Ser214 in trypsin. Whereas in trypsin the introduction of an amide disrupts hydrogen bonding, the situation in  $\Delta D99N$  PLA<sub>2</sub> is such that introduction of the amide group of asparagine requires only minimal structural changes to create hydrogen bonds where the amide functions as the donor and the hydroxyl group of Tyr73 and a water molecule as acceptors. Therefore, we propose that the hydrogen bond between the N3 atom of His48 and the carbonyl oxygen of Asn99 could remain intact in  $\Delta D99N$  PLA<sub>2</sub> (Figure 2B). In this model possible steric conflicts are negligible since only minor rearrangements of two hydrogen atoms are required. If this model is correct it means that the Asn99 residue in the  $\Delta D99N$  mutant is still able to stabilize the imidazole of His48 in the correct

tautomeric state for acid/base catalysis. This might explain why the decrease in enzymatic activity of the  $\Delta D99N$  PLA<sub>2</sub> is far less pronounced than the  $>10^4$  times reduced rate of the D102N trypsin mutant. The loss of enzymatic activity of  $\Delta D99N$  PLA<sub>2</sub> on micellar substrates may be ascribed partially to differences in the proximity of the reactive groups of the substrate and the enzyme, due to a slightly different active site architecture resulting from the removal of a negative charge from the interior of the enzyme. However, a more likely explanation is that the replacement of Asp99 by a noncharged Asn residue leads to a reduction of the negative potential on His48, which could destabilize the transition state.

A structural analysis of the mutants described in this paper might help to understand better side-chain interactions of these mutants. Therefore, attempts at crystallization of these mutants are being made currently. Awaiting these structures also, experiments which combine the mutations at positions 52, 73 and 99 might provide a means to shed more light on a possible cooperativity between these residues. The orienting capacity of Asn99, which is assisted by hydrogen bonds from Tyr52 and Tyr73 might be lost in mutants such as  $\Delta D99N/Y52F$  or  $\Delta D99N/Y73F$ , which makes their future production desirable.

### Note added in proof

After this manuscript was accepted for publication a paper appeared [Dupureur, C.M., Deng, T., Kwak, J.-G., Noel, J.P. and Tsai, M.-D. (1990) *J. Am. Chem. Soc.*, **112**, 7074–7076] describing substitutions of Tyr52, Tyr73 and Asp99 in bovine pancreatic phospholipase A<sub>2</sub>. The conclusions of these authors concerning the role of both tyrosine residues are in line with our conclusions. The substitution of Asn for Asp99 decreased the  $k_{cat}/K_m$  ratio for dioctanoyllecithin micelles 1000-fold in the bovine enzyme, as opposed to the 25-fold decrease observed by us with the same substrate. The high residual activity (65%) described in this paper for hydrolysis of monomeric substrates excludes the possibility that the 4% residual activity on micelles is caused by a contamination of the D99N mutant with Asp99 phospholipase A<sub>2</sub>. This suggests an interesting difference in the active site stabilization of the bovine pancreatic phospholipase A<sub>2</sub> used by Dupureur *et al.* and the porcine pancreatic phospholipase A<sub>2</sub> used by us, where a surface loop of five amino acids was also deleted.

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